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The putative neurotransmitters, L-glutamate and norepinephrine were microinjected (50nl) unilaterally into the paraventricular nucleus of pentobarbital-anesthetized rats. L-glutamate (0.5M) elicited a rise in plasma ACTH, vasopressin, and oxytocin and a profound drop in heart rate that was accompanied by no significant change in blood pressure. The bradycardia was blocked by pretreatment with pentolinium (ganglionic blocker) and attenuated by pretreatment with atropine. Microinjection of norepinephrine (10^{-4}M) also elevated plasma ACTH but had no effect on heart rate or blood pressure. Microinjection of phenylephrine at 10^{-4}M also led to a rise in plasma ACTH, however microinjection of Clonidine did not.

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S/N 0102-LF-014-6603

October 1, 1988

Annual Report on Contract N00014-88-K-0059

PRINCIPLE INVESTIGATORS: Mary F. Dallman, Ph.D. and Daniel N. Darlington, Ph.D.

CONTRACTOR: The Regents of the University of California

START DATE: 1 October, 1987

OBJECTIVES: To determine whether the putative neurotransmitter L-glutamate and the class of neurotransmitters known as the catecholamines regulate adrenocorticotropin and vasopressin release and cardiovascular function directly at the level of the paraventricular nucleus; and if so, which receptor type is involved.

MATERIALS AND METHODS: Male Sprague-Dawley rats (250-400g) were anesthetized with pentobarbital-sodium (45mg/kg) and cannulas were placed in the femoral artery and vein (PE-50) for measurement of arterial pressure, heart rate and for blood withdrawal. The rats were placed in a stereotaxic device, the skin and muscle of the cranium retracted and a burr hole was drilled at the coordinates descsribed below. Each rat was allowed one hour to stabilize before any further manipulation. Pentobarbital was given as needed (2.6mg, intravenously) to maintain anesthesia; however, this dose also allowed elicitation of baroreflexes. A glass micropipette (approx. 50μm O.D.), filled with either freshly prepared L-glutamate, acetate, norepinephrine, phenylephrine or clonidine was lowered into the paraventricular nucleus (-1.4 to -1.8mm from bregma, 0.4mm lateral from midline and 7-7.5mm ventral to the dura at the top of the skull) or surrounding nuclear structures in the hypothalamus. Ten minutes later, 50nl of L-glutamate or acetate was microinjected over 2 minutes (WPI nanoliter pump). In

another experiment involving glutamate microinjections, 26 rats were pretreated 10 to 15 minutes before the second PVN microinjection with an intravenous injection of 2mg/kg atropine sulfate (Mallinckrodt), 1.5mg/kg propranolol hydrochloride (Ayerst) or 20mg/kg pentolinium tartrate (Aldrich) to chemically block parasympathetic, beta-adrenergic and ganglionic control of heart rate.

In some rats, L-glutamate, acetate, norepinephrine, phenylephrine or clonidine was injected unilaterally into each PVN and 1.0ml of venous blood was drawn before and varying times after microinjection. The red cells from each sample, suspended in saline, were returned to the rat before the next sample. Plasma ACTH, vasopressin or oxytocin concentrations were determined only from rats that had histologically verified injections in the PVN. A single hormone was determined in plasma from each rat.

Twenty one rats had monopolar electrodes (FHC electrodes, Brunswick,ME, 20μm tip uninsulated), placed in the PVN and were stimulated with a 10sec train of negative pulses (0.5msec duration, 2-500μA, 2-500Hz) delivered from a constant current isolation unit while measuring arterial blood pressure and heart rate. The greatest change in arterial blood pressure and heart rate was determined. The current intensity and frequency were always monitored on a oscilloscope. A lesion was made (50μA, DC current for 10sec) at the tip of the electrode for identification of the stimulation site. The coordinates for placement of the electrodes in the PVN were the same as described above.

Arterial blood pressure and heart rate were monitored continuously throughout each experiment.

At the conclusion of the experiment, each rat was perfused with heparinized saline followed by 10% formalin via the left cardiac ventricle. The brains were removed, stored in 10% formalin for not less than 2 days, serially sectioned at 30μm and stained with thionin for identification of the electrode tracts

and injection sites. Injection sites were located by identifying the lesion made by the micropipette in serial sections.

RESULTS: Using a new microinjection system which accurately delivers nanoliter volumes into specific nuclear regions, we have completed a preliminary study showing that activation of neurons in the paraventricular nucleus of the hypothalamus with the excitatory neurotransmitter, L-glutamate, leads to the release of adrenocorticotropin, vasopressin and oxytocin from the pituitary (Figure 1). Also, activation of these neurons led to a profound bradycardia with little change in arterial blood pressure (Figure 2). This response was dose and volume dependent (Figure 3) and mediated predominantly by the parasympathetic nervous system (Figure 4) presumably though projections of the paraventricular nucleus to the preganglionic parasympathetic neurons in the nucleus ambiquus and dorsal motor nucleus of the vagus. This effect could only be produced by glutamate injection into the paraventricular nucleus and not in any of the surrounding nuclei in the hypothalamus and thalamus (Figure 5). This finding was confirmed by focal electric stimulation of the paraventricular nucleus and surrounding nuclear structures (Figure 6). Electric stimulation of the paraventricular nucleus led to a decrease in heart rate that was dependent on current amplitude and frequency (Figure 7). This report has been accepted for publication in the American Journal of Physiology.

Conclusions: The paraventricular nuclei contain neurons that can be stimulated by glutamate and this stimulation leads to a rise in plasma ACTH, vasopressin and oxytocin and changes in heart rate. The changes in heart rate are mediated though the autonomic nervous system.

Using this microinjection technique that we have characterized above, we have begun microinjecting 50nl of norepinephrine, phenylephrine and clonidine (doses ranging from 10^{-9}, 10^{-8}, 10^{-7}, 10^{-6}, 10^{-5}, $10^{-4}$to 10^{-3}M, n>9 for each injection dose) directly into the paraventricular nucleus. Plasma was taken for determination of adrenocorticotropin. Arterial blood pressure and heart rate were monitored during and after injection. Not all plasma samples have been analyzed to date. However, at the present time, we have found that, unlike L-glutamate, microinjection of norepinephrine (10^{-9} to 10^{-4}M), phenylephrine (10^{-9} to 10^{-4}M) or clonidine (10^{-9} to 10^{-6}M) into the paraventricular nucleus had no effect on heart rate or arterial blood pressure. However, the higher doses of norepinephrine and the highest dose of phenylephrine that has been tested (10^{-4}M) have produced significant elevations of plasma ACTH (Figure 8). Microinjection of clonidine into the paraventricular nucleus has not produced any significant elevation in plasma ACTH (at the doses tested to this date). We have also found that the peak ACTH response to microinjection of 10^{-4}M norepinephrine occurs 2 to 5 minutes after injection (Figure 9) and that the response is the same whether the injection is made on the left or the right paraventricular nuclei (Figure 10).

Conclusions: At this time, microinjection of norepinephrine into the paraventricular nucleus appears to elevate plasma ACTH levels with no change in heart rate or blood pressure. It is too early to tell which receptor type (alpha-1, alpha-2 or beta) is involved.

Figure Legends

Figure 1. Plasma levels of ACTH, vasopressin and oxytocin in response to activation of cell bodies in the paraventricular nucleus (PVN) with L-glutamate. Right side of Figure shows corresponding heart rate changes. All glutamate-induced responses are significant and different from the acetate-induced responses. Values represent mean$\pm$SE. inj.- injection period. Numbers next to each graph represent the number of successful injections into the PVN.

Figure 2 shows the responses of mean arterial blood pressure (MABP) and heart rate to activation of cell bodies in the PVN by a 2 minute unilateral microinjection of 50nl of L-glutamate or acetate. The response of heart rate to microinjection of L-glutamate is significant and different from the response to acetate. Values represent mean$\pm$SE. Glutamate - n=39; Acetate - n=13

Figure 3. (top) The maximal change in heart rate to unilateral microinjection of varying concentrations of L-glutamate into the PVN. (bottom) The maximal change in heart rate to varying volumes of glutamate.

Figure 4. The effect of atropine, propranolol, pentolinium and vehicle (saline) pretreatment on the response of heart rate to unilateral microinjection of L-glutamate into the PVN. glu. - period of L-glutamate injection. Values represent mean$\pm$SE. Saline- n=26, atropine- n=8, propranolol- n=8, pentolinium- n=10.

Figure 5. The maximal change in mean arterial blood pressure (MABP) and heart rate to activation of cell bodies in nuclei around the PVN. MPO-medial preoptic area, AHA-anterior hypothalamic area, PVN-paraventricular nucleus, ZI-zona incerta, RE-nucleus reuniens, DMN-dorsal medial nucleus, VMN-ventral medial nucleus, LHA-lateral hypothalamic area. Values represent mean$\pm$SE. Q Significant response; + LHA response significantly different from all other responses; + + PVN response significantly different from all other responses.

Figure 6. Focal electric stimulation of the PVN and regions around that area. Notice that bradycardia is elicited only when the PVN is stimulated. n=14 rats, 2-4 stimulation tracks each.

Figure 7 shows the maximal change in mean arterial blood pressure (MABP) and heart rate during electric stimulation of the PVN while varying current intensity (n=8) and frequency (n=7). Values represent mean$\pm$SE.

Figure 8. Change in plasma ACTH after unilateral microinjection of varying concentrations of catecholamines into the PVN. Change is the difference between plasma ACTH determined before and 5 minutes after injection.

Figure 9. Plasma ACTH levels before and after unilateral microinjection of 10^{-4}M norepinephrine into the PVN. n represents the number of injections.

Figure 10. Plasma ACTH levels before and after microinjection of 10^{-4}M norepinephrine into either the left or right half of the PVN.

Fig 1

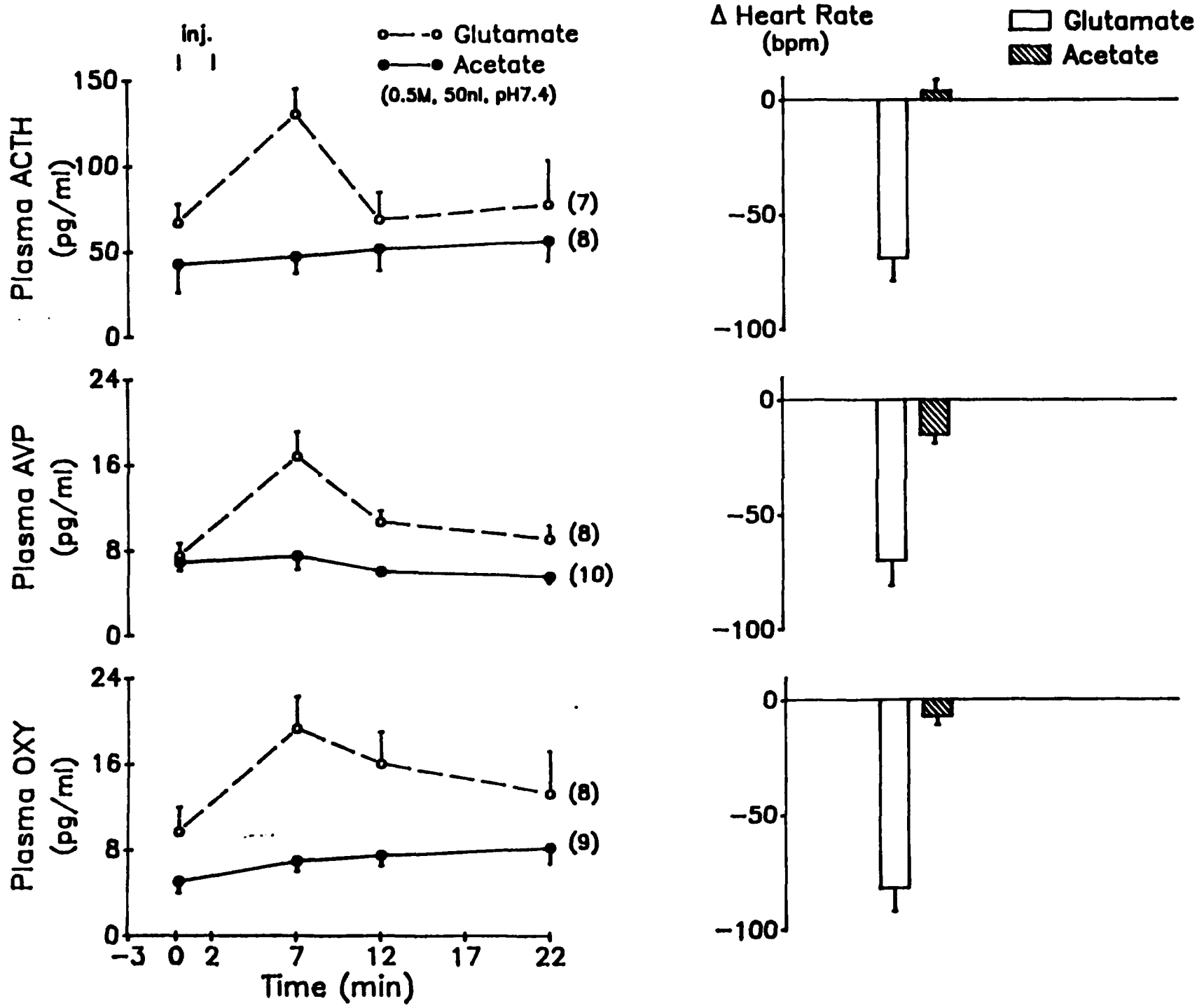

inj.
Glutamate
Acetate
(0.5M, 50nl, pH7.4)
Plasma ACTH (pg/ml)
150
100
50
0
(7)
(8)
Plasma AVP (pg/ml)
24
16
8
0
(8)
(10)
Plasma OXY (pg/ml)
24
16
8
0
(8)
(9)
−3 0 2 7 12 17 22
Time (min)
Δ Heart Rate (bpm)
Glutamate
Acetate
0
−50
−100
0
−50
−100
0
−50
−100

Fig 2

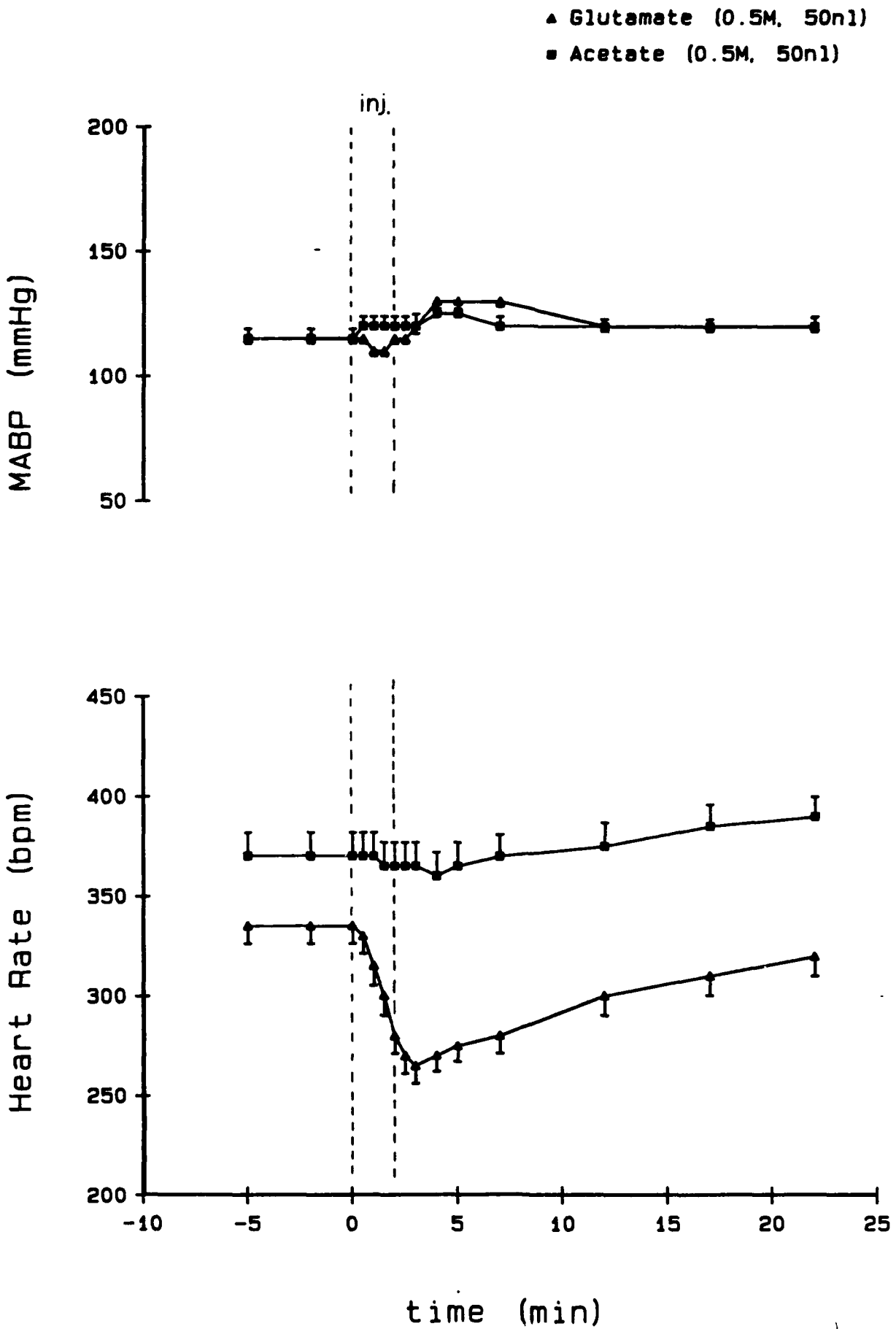
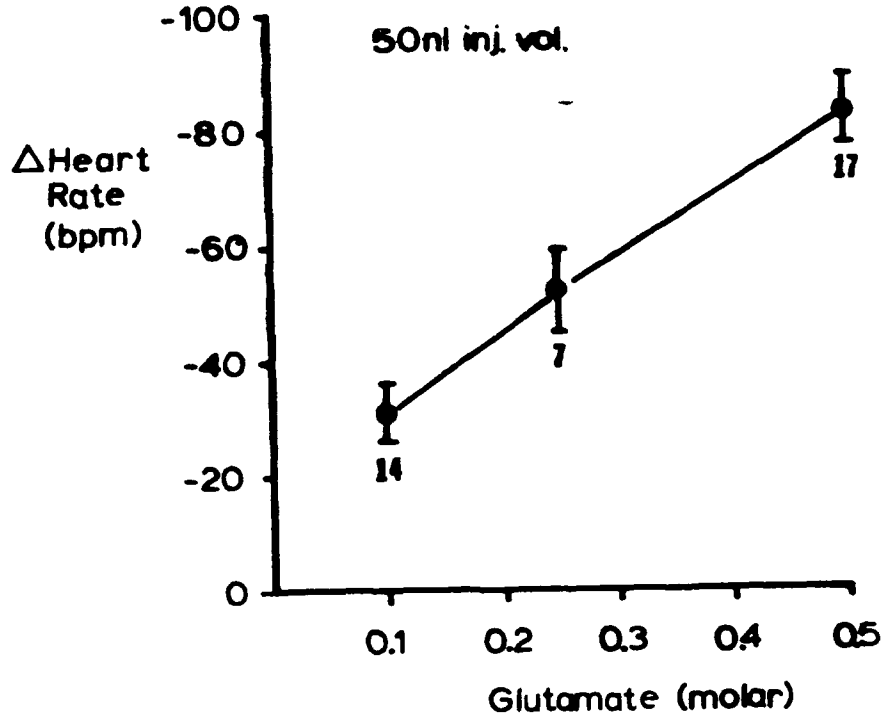

▲ Glutamate (0.5M, 50nl)
■ Acetate (0.5M, 50nl)
inj.
MABP (mmHg)
200
150
100
50
Heart Rate (bpm)
450
400
350
300
250
200
-10
-5
0
5
10
15
20
25
time (min)

Fig 3


50nl inj. vol.
ΔHeart Rate (bpm)
-100
-80
-60
-40
-20
0
0.1
0.2
0.3
0.4
0.5
Glutamate (molar)
14
7
17

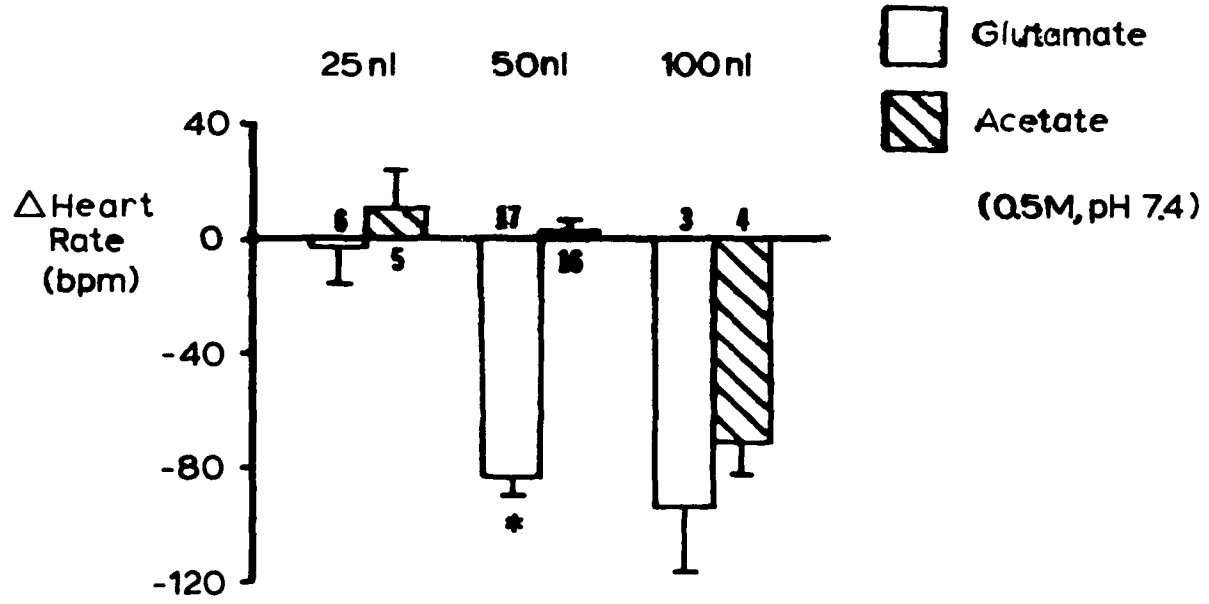

25nl
50nl
100nl
Glutamate
Acetate
(0.5M, pH 7.4)
ΔHeart Rate (bpm)
40
0
-40
-80
-120
6
5
17
16
3
4
*

Figure 4

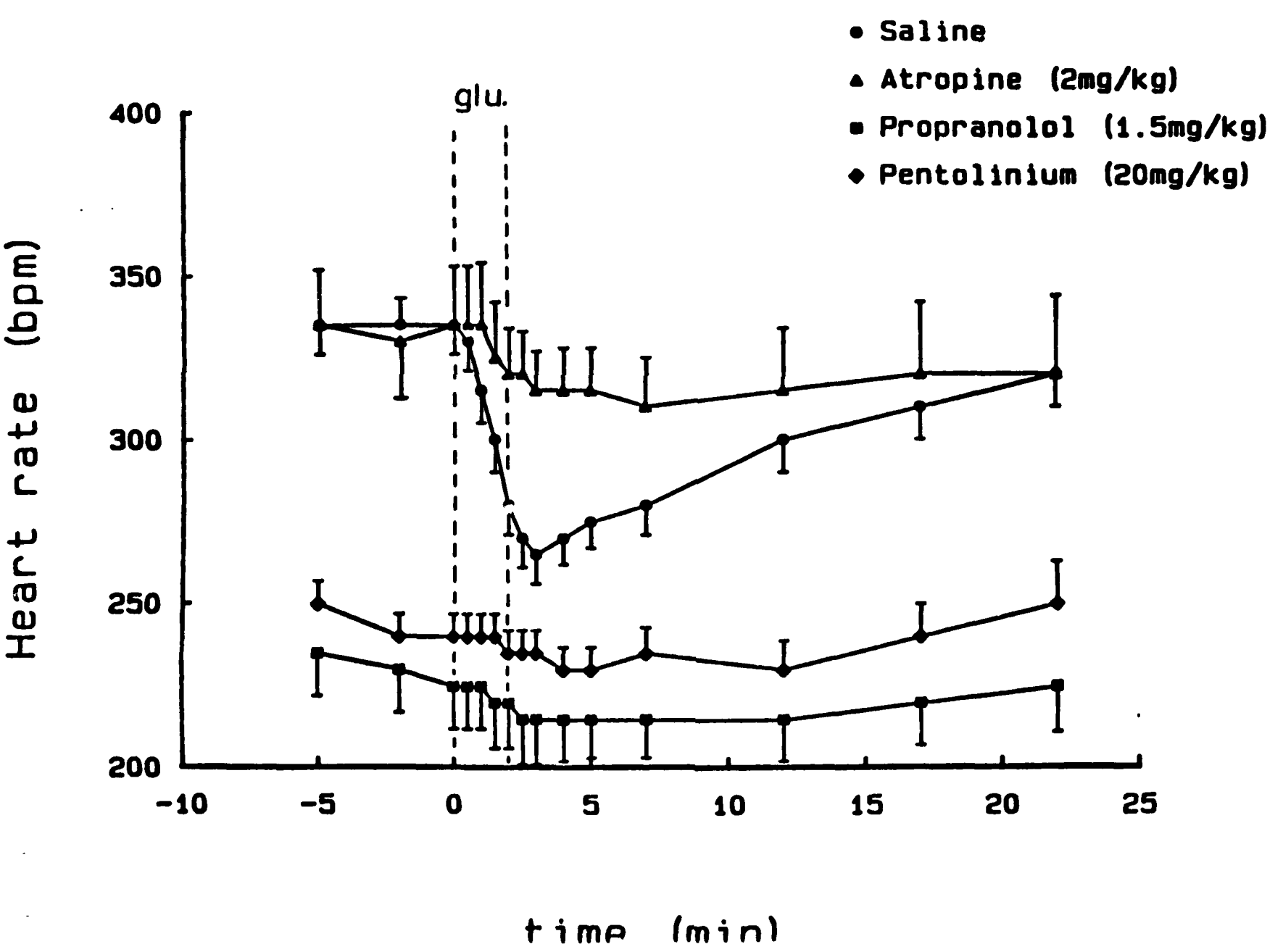
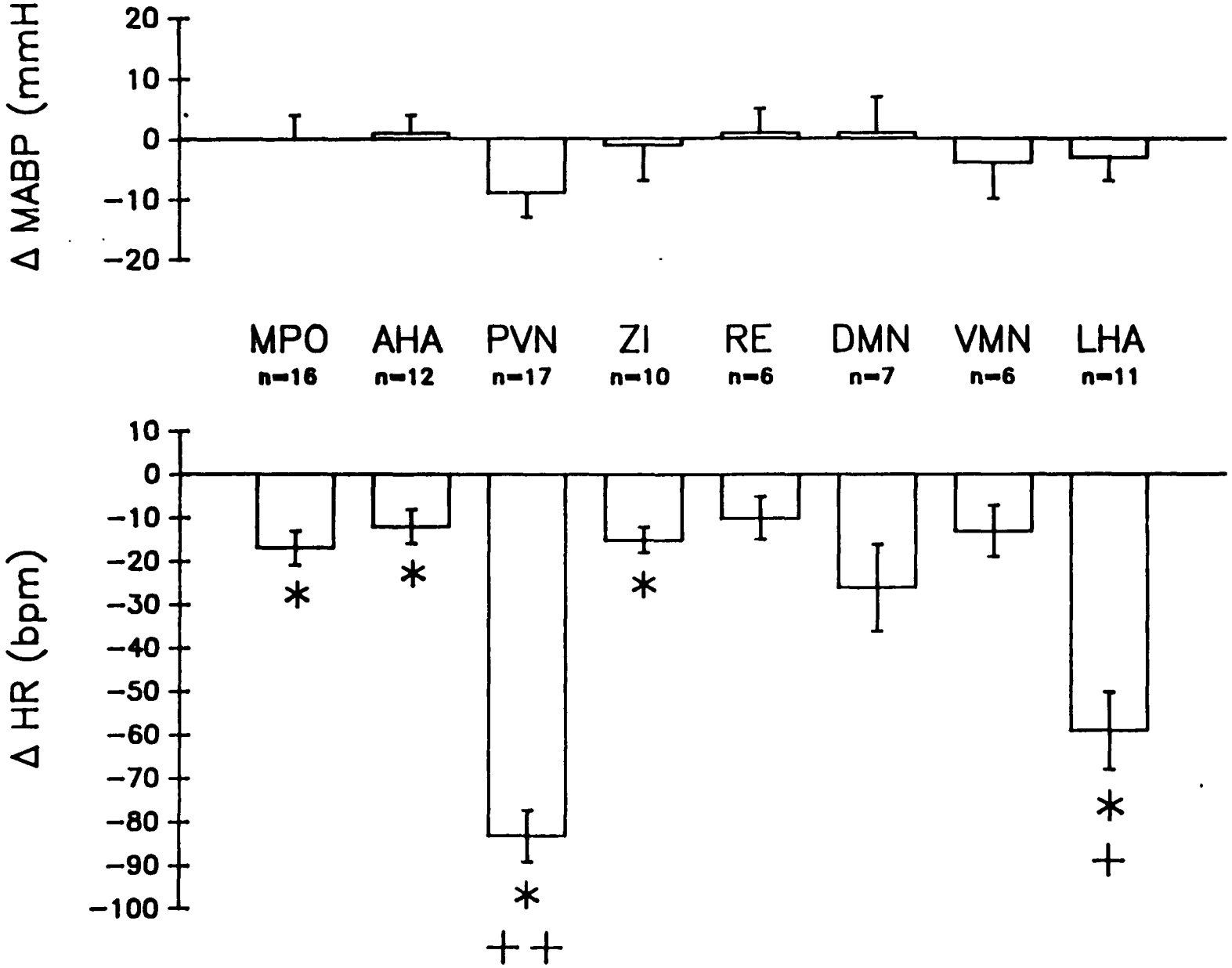

• Saline
▲ Atropine (2mg/kg)
■ Propranolol (1.5mg/kg)
◆ Pentolinium (20mg/kg)
glu.
Heart rate (bpm)
400
350
300
250
200
-10
-5
0
5
10
15
20
25
time (min)

Figure 5

Responses of MABP and HR to microinjection (50 nl) of L–Glutamate (0.5M) into hypothalamic nuclei.



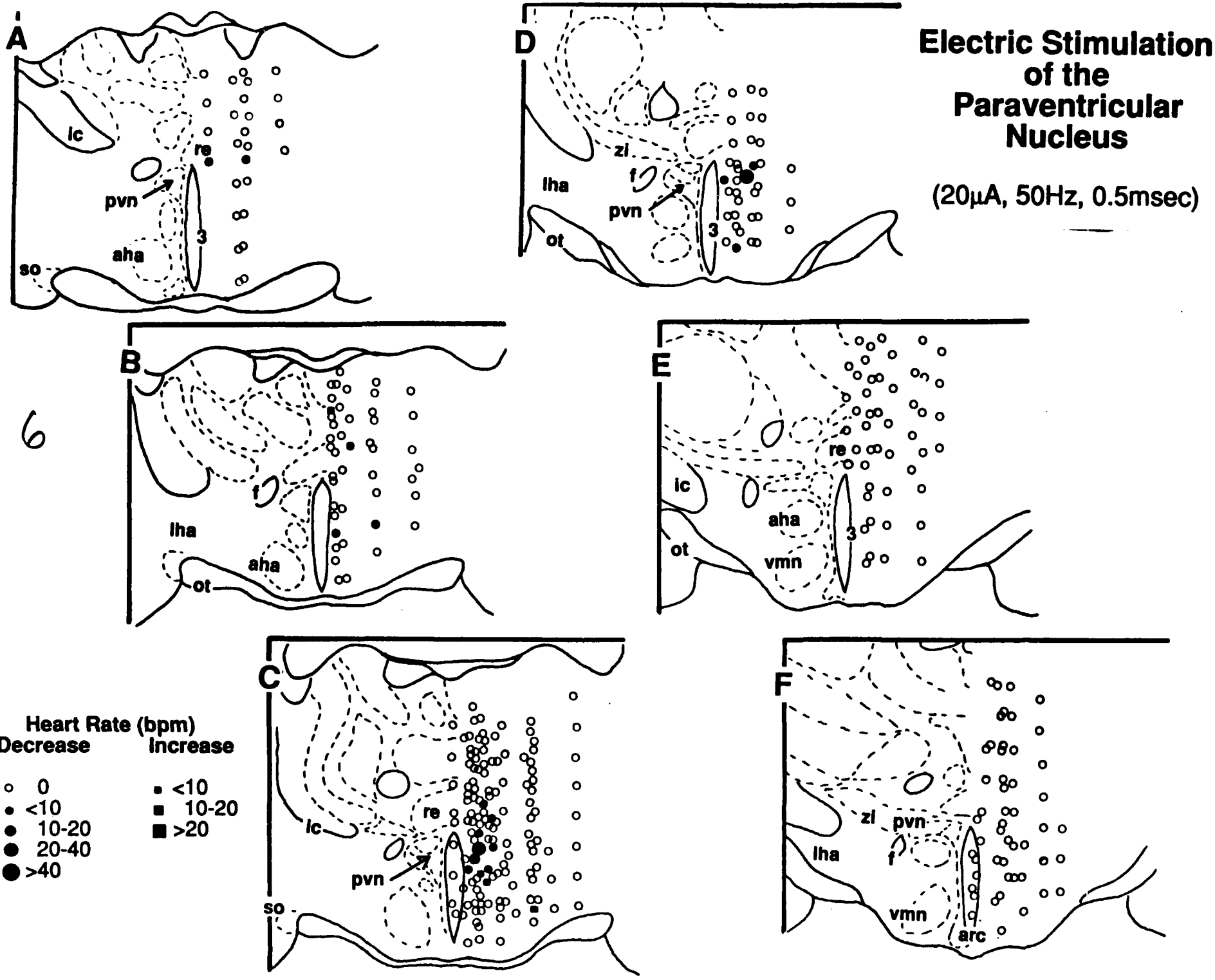

A
ic
re
pvn
3
aha
so
D
zl
f
lha
pvn
3
ot
Electric Stimulation of the Paraventricular Nucleus
(20μA, 50Hz, 0.5msec)
B
f
lha
aha
ot
E
re
ic
aha
3
ot
vmn
C
re
ic
pvn
so
F
zl
pvn
f
lha
vmn
arc
Heart Rate (bpm)
Decrease
0
<10
10-20
20-40
>40
Increase
<10
10-20
>20

Fig 6

Fig 7

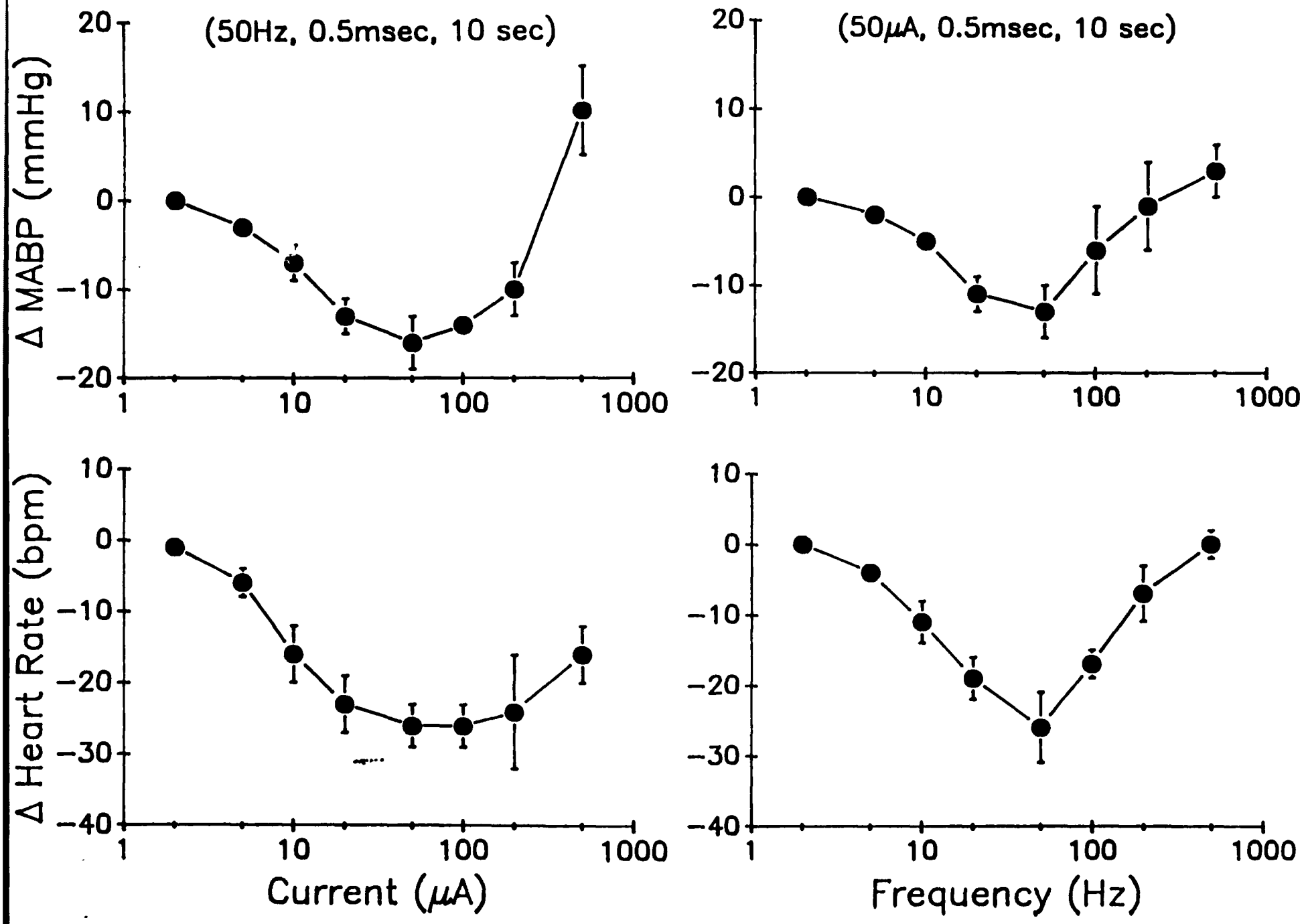

(50Hz, 0.5msec, 10 sec)
(50μA, 0.5msec, 10 sec)
Δ MABP (mmHg)
Δ Heart Rate (bpm)
20
10
0
−10
−20
−30
−40
1
10
100
1000
Current (μA)
Frequency (Hz)

Figure 8

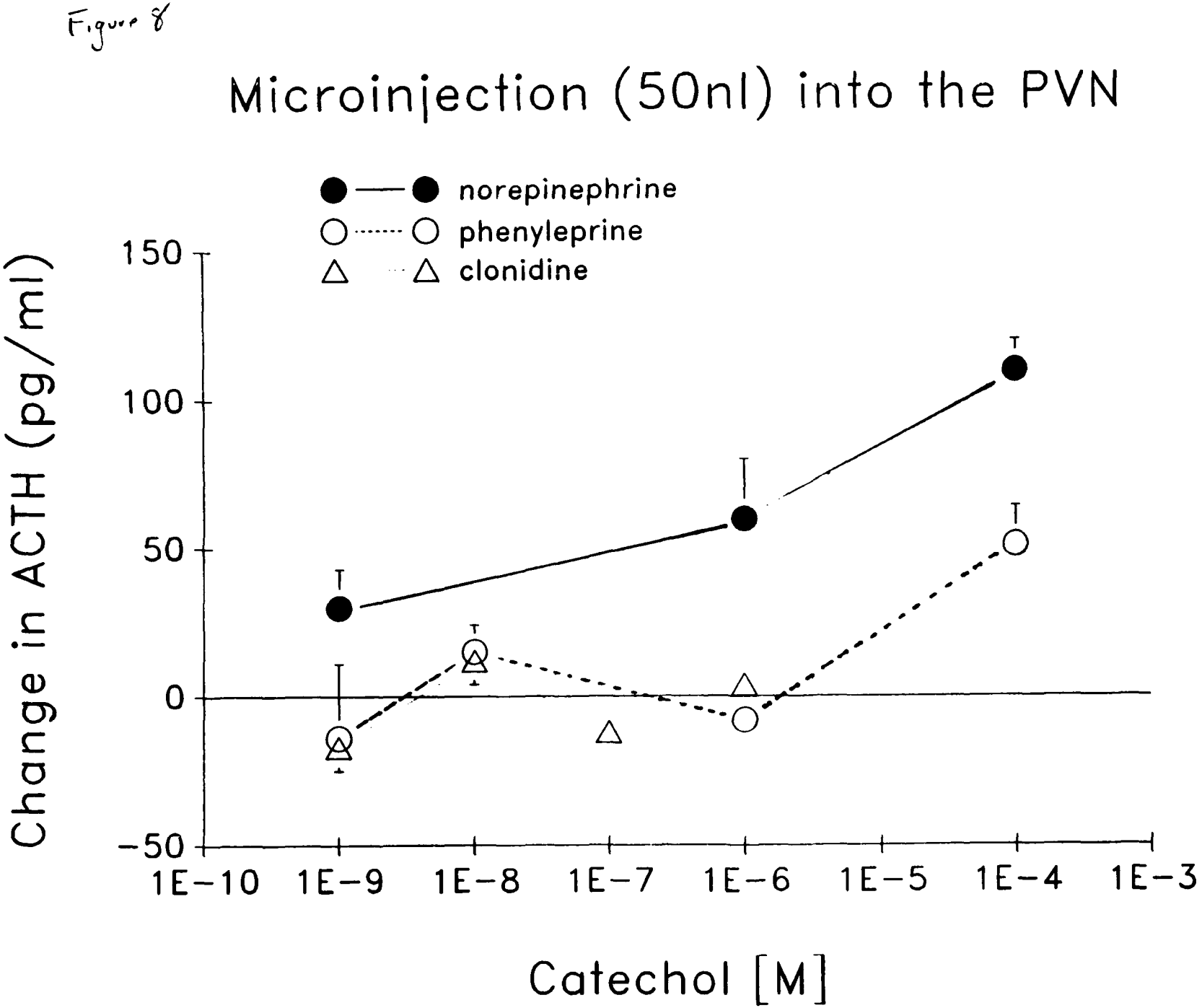

Microinjection (50nl) into the PVN
norepinephrine
phenyleprine
clonidine
Change in ACTH (pg/ml)
150
100
50
0
-50
1E-10
1E-9
1E-8
1E-7
1E-6
1E-5
1E-4
1E-3
Catechol [M]

Fig 9

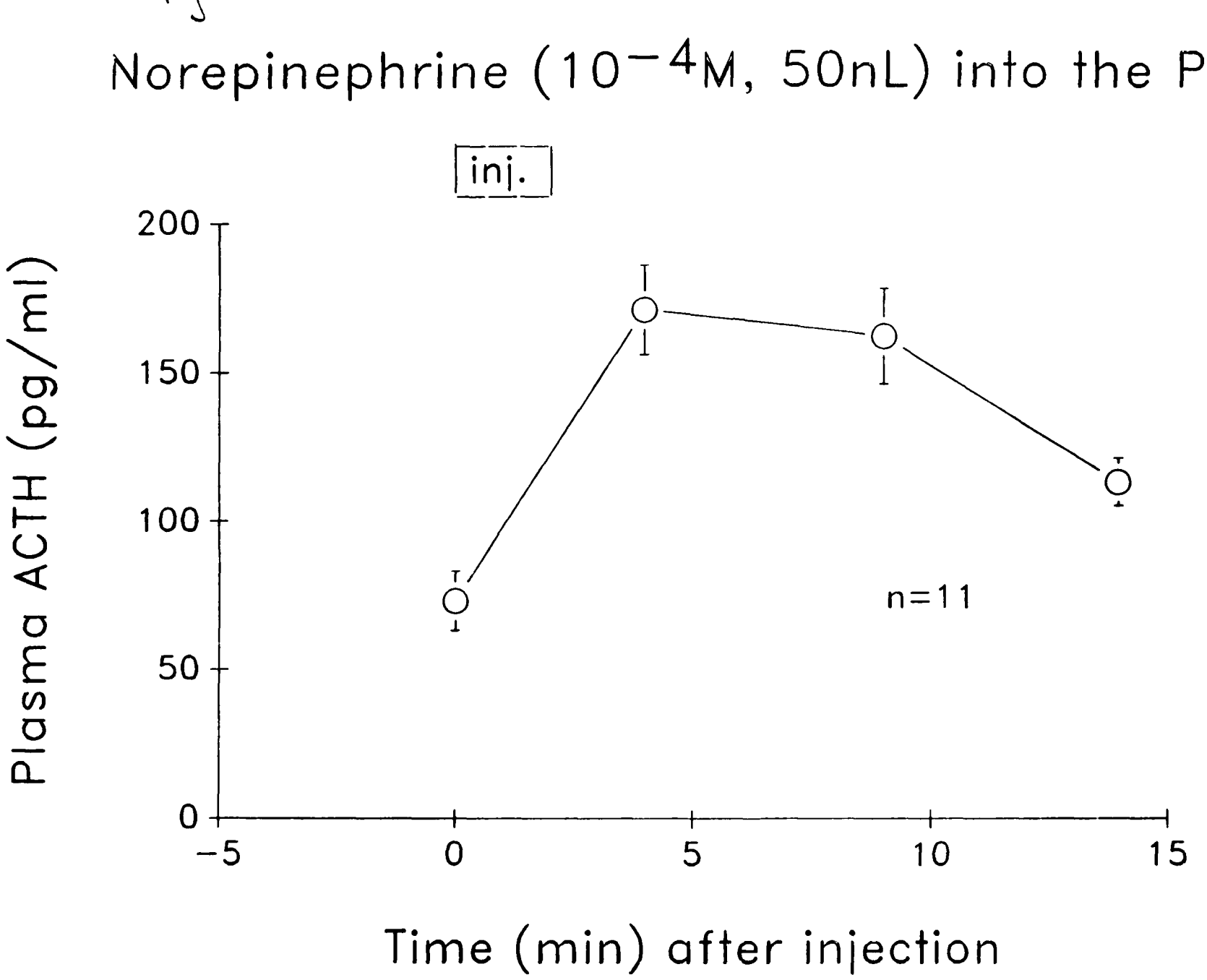
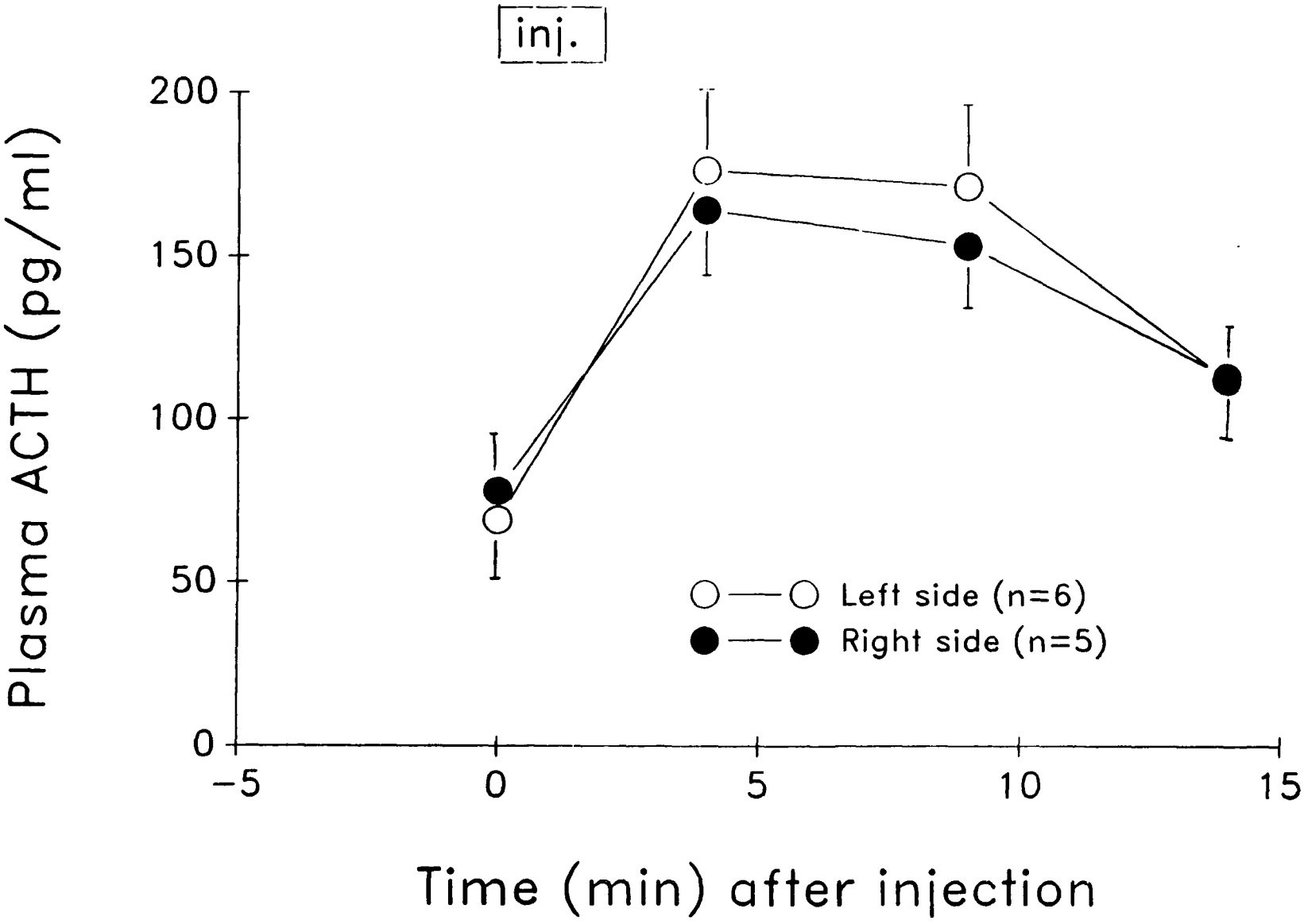

Norepinephrine (10^{-4}M, 50nL) into the PVN
inj.
200
150
100
50
0
Plasma ACTH (pg/ml)
n=11
−5
0
5
10
15
Time (min) after injection

Fig 10


inj.
200
150
100
50
0
Plasma ACTH (pg/ml)
−5
0
5
10
15
Left side (n=6)
Right side (n=5)
Time (min) after injection